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Neural stem/progenitor cells display a low requirement for oxidative metabolism independent of hypoxia inducible factor-1 alpha expression

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Abstract

Neural stem/progenitor cells (NSPCs) are multipotent cells within the embryonic and adult brain that give rise to both neuronal and glial cell lineages. Maintenance of NSPC multi-potency is promoted by low oxygen tension, although the metabolic underpinnings of this trait have not been described. In this study, we investigated the metabolic state of undifferentiated NSPCs in culture, and tested their relative reliance on oxidative versus glycolytic metabolism for survival, as well as their dependence on hypoxia inducible factor-1alpha (HIF-1 α) expression for maintenance of metabolic phenotype. Unlike primary neurons, NSPCs from embryonic and adult mice survived prolonged hypoxia in culture. In addition, NSPCs displayed greater susceptibility to glycolytic inhibition compared with primary neurons, even in the presence of alternative mitochondrial TCA substrates. NSPCs were also more resistant than neurons to mitochondrial cyanide toxicity, less capable of utilizing galactose as an alternative substrate to glucose, and more susceptible to pharmacological inhibition of the pentose phosphate pathway by 6-aminonicotinamide. Inducible deletion of exon 1 of the *Hif1a* gene improved the ability of NSPCs to utilize pyruvate during glycolytic inhibition, but did not alter other parameters of metabolism, including their ability to withstand prolonged hypoxia. Taken together, these data indicate that NSPCs have a relatively low requirement for oxidative metabolism for their survival and that hypoxic resistance is not dependent upon HIF-1 α signaling.

Keywords

glycolysis; HIF; hypoxia; metabolism; neural progenitor cell; neural stem cell

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The authors of this manuscript declare no conflict of interest.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Adult NSPCs display a similar metabolic phenotype as embryonic NSPCs.

Figure S2. NSPCs are immunopositive for the cell cycle marker Ki67.

Figure S3. Deletion of *Hif1a* does not alter the metabolic phenotype of adult NSPCs.

Figure S4. Deletion of *Hif1a* does not alter protein expression of HIF-2 α .

Neural stem/progenitor cells (NSPCs) are multipotent cells with self-renewing capabilities. During embryonic development, radial glial cells within the ventricular zone serve as primitive neural stem cells that give rise to both neuronal and glial lineages [for review, see (Kriegstein and Alvarez-Buylla 2009)]. Neural stem/progenitor cells derived from embryonic radial glia persist throughout adulthood within restricted regions that include the forebrain subventricular zone (SVZ) and hippocampal dentate subgranular zone (SGZ), where they continue to give rise to new neurons of the adult olfactory bulb and dentate gyrus, respectively (Gage 2000; Doetsch 2003a,b). Although the role of post-natal neurogenesis in brain function remains controversial, the potential therapeutic use of NSPCs to replace damaged neurons and glia within the central nervous system is an area of intense research interest and clinical significance. Therapeutic approaches include transplantation of neural stem cells that have been expanded in an undifferentiated state *ex vivo*, and manipulation of endogenous NSPCs that are resident within the post-natal brain. The therapeutic significance of understanding neural stem cell biology is also underscored by a potential link with CNS cancer stem cells, which share many properties with normal adult neural stem cells (Germano *et al.* 2010; Mohyeldin *et al.* 2010). Finally, endogenous NSPCs display a remarkable capacity to withstand many types of metabolic and traumatic brain injuries and to initiate an endogenous regenerative response (Lichtenwalner and Parent 2006), making them attractive targets for promoting structural and functional brain repair.

Metabolic state is likely to influence the maintenance of the stem cell pool and NSPC survival following traumatic and metabolic insults. Oxygen tension has been shown to influence neural stem cell properties during normal development and disease (Morrison *et al.* 2000; Studer *et al.* 2000; Panchision 2009; Mohyeldin *et al.* 2010). In culture, low oxygen tension promotes NSPC self-renewal, stimulates proliferation and alters phenotypic outcome following differentiation. *In vivo*, disruption of oxygen availability to the brain during early post-natal development (Yang *et al.* 2008) or in adulthood (Arvidsson *et al.* 2002; Thored *et al.* 2006; Li *et al.* 2010) promotes cellular proliferation within the SVZ and stimulates migration of cells derived from the SVZ into the hypoxic brain region. Although several studies have demonstrated that NSPCs thrive under low oxygen conditions (Morrison *et al.* 2000; Studer *et al.* 2000; Santilli *et al.* 2010), the metabolic underpinnings of this property have not been delineated.

Hypoxia Inducible Factor-1 α (HIF-1 α) is part of the HIF-1 transcriptional complex involved in regulation of target genes associated with hypoxic adaptation (Semenza 2012). In most cell types, HIF-1 α protein is constitutively produced but rapidly degraded in the presence of oxygen. Under hypoxic conditions, HIF-1 α protein is stabilized and translocates to the nucleus to dimerize with HIF-1 β (which is not susceptible to oxygen-dependent degradation) to form the HIF-1 transcriptional complex. This complex binds to response elements in genes encoding metabolic enzymes and transporters that promote glycolytic, over oxidative, metabolism. NSPCs express a basal level of stabilized HIF-1 α under normoxic conditions in culture and within the subventricular and subgranular zones of post-natal mouse brain *in vivo* (Mazumdar *et al.* 2010; Roitbak *et al.* 2011). Constitutive stabilization of HIF-1 α under normoxic conditions in NSPCs suggests that HIF-1 α might play a role in regulating metabolism under both normoxic and hypoxic conditions. To test this hypothesis, we evaluated the metabolic phenotype of embryonic and adult NSPCs by

assessing their relative dependence on glycolysis *versus* mitochondrial oxidative phosphorylation for survival. We then investigated the role of *Hif1a* gene expression on the maintenance of NSPC metabolic state, using a conditional, tamoxifen-inducible Cre-loxP approach to generate NSPCs that harbor bi-allelic deletion of exon 1 of the *Hif1a* gene. We demonstrate that NSPCs display a relatively low requirement for oxidative metabolism for their survival in culture, and show that this metabolic trait is independent of endogenous HIF-1 α expression.

Materials and methods

Primary cell culture

This study was approved by the University of New Mexico Animal Care and Use Committee and conformed to the NIH Guidelines for use of animals in research. Timed pregnant female mice were euthanized by isoflurane overdose and the embryos removed by cesarean section.

Neural stem/progenitor cell cultures—Wild-type NSPCs were isolated from embryonic day 14.5 (E14.5) or post-natal day 51 (PD51) C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA). Briefly, embryonic NSPCs (eNSPC) were established from whole telencephalon as previously described (Harms *et al.* 2010). Adult NSPCs (aNSPC) were established from microdissected SVZ of PD51 male C57BL/6J mice using described protocols in conjunction with the MACS Neural Tissue Dissociation Kit (Miltenyi Biotec, Auburn, CA, USA) (Roitbak *et al.* 2008). Following dissociation, NSPCs were plated in six-well tissue culture plates pre-coated with poly-L-lysine (BD Biosciences, San Diego, CA, USA). The cultures were maintained under serum-free conditions in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with B-27 supplement (2%; Invitrogen), glutamine (2.0 mM; Sigma, St Louis, MO, USA), penicillin (100 U/mL; Invitrogen), streptomycin (100 μ g/mL; Invitrogen), epidermal growth factor (EGF; 10 ng/mL; Invitrogen), and basic fibroblast growth factor (bFGF; 20 ng/mL; Invitrogen). Growth factors (EGF and bFGF) were added every other day and cells were passaged two to eight times prior to use.

For experiments, NSPCs were subcultured on poly-L-lysine-coated glass coverslips within 24-well culture plates at a density of 3.125×10^4 cells/cm². We have previously shown that these cultures are uniformly nestin-positive and undergo spontaneous differentiation into neurons and glia after growth factor withdrawal (Roitbak *et al.* 2008; Harms *et al.* 2010).

Hif1a gene deletion using nestin-CreER^{T2}/R26R-YFP/Hif1a^{fl/fl} mice—*Nestin*-CreER^{T2}/R26R-YFP/*Hif1a*^{fl/fl} mice were generated by crossing the *nestin*-CreER^{T2}/R26R-YFP transgenic line [generously provided by Dr. Amelia Eisch, UTSW Medical Center, Dallas, TX, USA; (Lagace *et al.* 2007)] with mice harboring floxed exon 1 of the *Hif1a* gene locus (*Hif1a*^{fl/fl}) [The Jackson Laboratory; (Ryan *et al.* 2000)], both on the C57BL/6J background. Offspring were bred to homozygosity at all three transgenes. To induce Cre-mediated recombination in nestin-positive embryonic NSPCs, tamoxifen was administered to *nestin*-CreER^{T2}/R26R-YFP/*Hif1a*^{wt/wt} (control) or *nestin*-CreER^{T2}/R26R-YFP/*Hif1a*^{fl/fl} timed-pregnant females at gestational day 10.5 (E10.5; 75 mg/kg tamoxifen dissolved in

10% ethanol/90% sunflower oil; Sigma; 2 injections i.p. spaced 6 hours apart), as previously described (Battiste *et al.* 2007). Embryos were collected on E14.5 and NSPCs were isolated from embryonic telencephalon as described above. To induce Cre-mediated recombination in nestin-positive post-natal NSPCs, tamoxifen was administered to young adult *nestin-CreER^{T2}/R26R-YFP/Hif1a^{wt/wt}* (control) or *nestin-CreER^{T2}/R26R-YFP/Hif1a^{fl/fl}* male mice (post-natal day 41; 180 mg/kg tamoxifen per day, i.p., for 5 consecutive days). Five days following the final tamoxifen injection, the mice were killed by isoflurane overdose and NSPCs were isolated from the SVZ as described above. Following the second passage, YFP-positive NSPCs from both genotypes were isolated using flow cytometry (ex/em 488 nm/530 nm; Beckman Coulter Legacy MoFlo, Brea, CA, USA), plated and expanded for 1–2 more passages prior to use in experiments. Excision of *Hif1a* exon 1 was confirmed by PCR using DNA isolated from NSPCs (forward primer 5'-TGT TAA ATA AAA GCT TGG AC -3' and reverse primer 5'-GCA GTT AAG AGC ACT AGT TG -3') (Ryan *et al.* 2000; Harms *et al.* 2010). Successful DNA recombination yields *Hif1a* gene-deleted NSPCs (*nestin-CreER^{T2}/R26R-YFP/Hif1a^{-/-}*), as indicated by the loss of the 1200 bp band amplification product and appearance of a 250 bp band (Fig. 5a).

Primary cortical neuron cultures—Primary neuronal cultures were established from cerebral cortices of C57BL/6J embryos on gestational day 15.5 (E15.5), using enzymatic dissociation with trypsin as previously described (Wetzel *et al.* 2008; Harms *et al.* 2010). For cell viability assays, 3.9×10^5 dissociated cortical cells were plated on poly-L-lysine-coated coverslips (0.1 mg/mL; Sigma) in 24-well plates (2.0×10^5 cells/cm²). For biochemical procedures, 1.95×10^6 cells were plated on pre-coated poly-L-lysine six-well plates (2.0×10^5 cells/cm²; BD Biosciences). The cultures were maintained under serum-free conditions in Neurobasal medium (Invitrogen, Carlsbad, CA, USA), supplemented with B-27 supplement (2%; Invitrogen), glutamine (0.5 mM; Sigma), glutamate (25 μ M; Sigma), penicillin (100 U/mL), and streptomycin (100 μ g/mL; Invitrogen). At 4 days *in vitro*, half of the medium was removed and replaced with fresh medium without glutamate. At 7 days *in vitro*, neuronal cultures were used for experimentation. All cultures were maintained in a humidified incubator at 37°C with 5% CO₂.

Oxygen deprivation

For oxygen deprivation, primary NSPCs or cortical neuronal cultures were placed in an anaerobic chamber (Coy Laboratories, Grass Lake, MI, USA) containing a gas mixture of 5% CO₂, 5% H₂, and 90% N₂ (< 0.2% O₂) for 24 h. Normal culture media was replaced with deoxygenated culture media. Media was supplemented as described above for the appropriate cell type. Sister cultures placed at 37°C under normoxic (21% O₂) conditions served as non-hypoxic negative controls. Cell viability was normalized to non-hypoxic controls in all experiments.

Pharmacological treatments

Neural stem/progenitor or neuronal cultures were exposed for 24 h to the following compounds dissolved in culture media: sodium cyanide (CN; 1.0 mM; Sigma); 6-aminonicotinamide (6-AN; 500 μ M; Sigma) or vehicle control (dimethylsulfoxide; vol/vol; Sigma). For addition of the following compounds, NSPC or neuronal cultures were

incubated in glucose- and pyruvate-free Neurobasal media (Invitrogen) supplemented as described above for the appropriate cell type: glucose (10 mM; Sigma) and pyruvate (0.1–10 mM; Sigma) with or without 2-deoxy-D-glucose (2-DG; 10 mM; Sigma); galactose (25 mM; Sigma) or glucose (25 mM).

Cell viability assay

Cell viability was assessed using the Calcein AM component of the Live/Dead Viability Cytotoxicity Kit for mammalian cells (Invitrogen). Cultured cells were washed twice with sterile Dulbecco's Phosphate Buffered Saline (Invitrogen) before incubation in 3 μ M Calcein AM for 45 min at 37°C in the presence or absence of oxygen (21%). Fluorescence was read at ex/em 494 nm/517 nm using background subtraction of fluorescence from cell-free wells containing Calcein AM. Viability was verified using meth-ylthiazolyldiphenyl-tetrazolium bromide dissolved in sterile Dulbecco's Phosphate Buffered Saline (0.5 mg/mL; Sigma) as previously described (Harms *et al.* 2010) (data not shown).

Immunocytochemistry

Cultured cells were fixed on coverslips with 4% paraformaldehyde in 0.1 M sodium phosphate buffer and incubated in monoclonal mouse anti-NeuN (1: 200; Chemicon, Temecula, CA, USA), monoclonal mouse anti-Nestin (1: 500; Chemicon), and/or polyclonal rabbit anti-Ki67 (1: 500; Leica, Buffalo Grove, IL, USA). Immunofluorescence was visualized using FITC- or Cy3-conjugated secondary antibodies (1: 250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). 4',6-diamidino-2-phenylindole (DAPI) nuclear stain was used to identify cell bodies of cultured cells (300 nM; Invitrogen; not shown). To detect mitochondria, cells were incubated with Mito Tracker[®] Red CMXRos (200 nM; Invitrogen) before fixation. Fluorescence was visualized using high-resolution confocal microscopy (Zeiss LSM510, Thornwood, NY, USA) or conventional fluorescence microscopy.

Lactate dehydrogenase, lactate, and glucose colorimetric assays

For all colorimetric assays, NSPC or neuronal cultures were incubated in phenol red-free culture media for 24 h prior to assay measurements. Intracellular lactate dehydrogenase (LDH) activity was measured using the CytoTox96[®] Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol, and absorbance measured at 490 nm. Lactate concentration was measured in culture media using a colorimetric assay (BioVision, Mountain View, CA, USA), measuring absorbance at 570 nm. Lactate and LDH measurements were normalized to protein levels using the MicroBCA kit (Pierce, Rockford, IL, USA). Glucose was measured in fresh culture media and then again after incubation with cells in culture for 24 h (GAGO-20 kit; Sigma), according to the manufacturer's protocol.

Transmission electron microscopy

Transmission electron microscopy was performed as described (Roitbak *et al.* 2011). Briefly, cells were post-fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, osmicated in 0.5% OsO₄ phosphate buffer, dehydrated and infiltrated with EmBed 812 (Electron

Microscopy Sciences, Hatfield, PA, USA), polymerized at 60°C for 24 h, and stained with uranyl acetate. Digital images of 70 nm thin sections were acquired on a Hitachi H-7500 transmission electron microscope (Hitachi High-Technologies Science America, Inc., Northridge, CA, USA).

Statistical analyses

Data are expressed as means $\pm$ SEM. Significant differences between means were determined by student's *t*-test or two-way ANOVA with Bonferroni multiple comparisons *post hoc* analysis using Prism software (Graphpad Software, San Diego, CA, USA). *P*-values < 0.05 are considered statistically significant.

Results

Embryonic NSPCs are resistant to anoxia and mitochondrial inhibition

To investigate the relative dependence of embryonic NSPCs (eNSPCs) on oxidative metabolism for survival, we first compared the viability of eNSPCs to that of embryonic cortical neurons following 24 h exposure to oxygen deprivation ($< 0.2\% \text{ O}_2$). As shown in Fig. 1a, approximately 70% of eNSPCs survived 24 h anoxia, whereas only 27% of neurons survived. The morphology of neurons and eNSPCs under normoxic and anoxic conditions was visualized using immunofluorescence (Fig. 1b–e). To assess dependence on mitochondrial ATP synthesis for survival, we compared survival of neurons and eNSPCs following 24 h exposure to the mitochondrial toxin sodium cyanide (1.0 mM; CN), an inhibitor of cytochrome c oxidase (complex IV) activity. As shown in Fig. 2a, eNSPCs also displayed increased resistance to CN toxicity when compared with primary cortical neurons (82% vs. 55% survival, respectively, $p < 0.001$), further indicating a relatively low reliance on oxidative metabolism for NSPC survival. Adult NSPCs responded similarly to oxygen-deprivation and cyanide treatment (Figure S1).

Neural stem/progenitor cells have a high glycolytic requirement compared to primary neurons

To explore the extent to which eNSPCs are reliant on the anaerobic glycolytic arm of glucose metabolism for energy production and survival, eNSPCs were grown for 24 h under conditions of glycolytic inhibition in the presence of the tricarboxylic acid (TCA) cycle substrate, pyruvate. 2-deoxy-D-glucose (2-DG; 10 mM) was added to glucose-containing media (10 mM) in the presence of pyruvate (0.1–10 mM). 2-deoxy-D-glucose is a competitive inhibitor of glycolysis (Pelicano *et al.* 2006), and pyruvate serves as an oxidation substrate for ATP synthesis through oxidative phosphorylation. Normally, pyruvate can rescue cells from glycolytic inhibition, depending on the cell's ability to utilize oxidative phosphorylation for ATP production. As shown in Fig. 2b, incubation with 0.1 mM pyruvate rescued approximately 80% of cortical neurons from glucose deprivation, but was not sufficient to support the survival of eNSPCs. When the concentration of pyruvate was increased to doses of 1 mM and 10 mM, neuronal survival increased in a dose-dependent manner, whereas eNSPC viability remained drastically low (Fig. 2b). Adult NSPC viability was also impaired with pyruvate replacement of glucose (1 mM; Figure S1).

To further probe for the requirement of glycolysis, we assessed NSPC and neuronal viability following 24 h incubation in media containing galactose instead of glucose. Galactose enters the glycolytic pathway at the same level as glucose, but results in no net ATP synthesis via glycolysis and thereby forces use of alternative substrates, such as glutamine, as an energy source for mitochondrial metabolism. This scenario also leaves the pentose phosphate pathway intact (Weinberg and Chandel 2009). As shown in Fig. 2c, viability of primary cortical neurons was unaffected by galactose substitution. Although eNSPC viability was more impaired compared with primary neurons, viability was only reduced by approximately 23% when galactose was substituted for glucose in the culture media. These data suggest that the high glycolytic requirement of eNSPCs is not due entirely to the need for anaerobic glycolytic ATP production.

Whereas our cultured neurons are post-mitotic and express the neuronal marker NeuN (Harms *et al.* 2010), neural progenitor cells proliferate in culture and undergo continual mitosis as visualized using the cell cycle marker Ki67 (Figure S2). Previous studies have shown that proliferating cells have a relatively high glucose requirement because of high pentose phosphate pathway (PPP) activity (Vander Heiden *et al.* 2009; Lunt and Vander Heiden 2011). To test the relative dependence of eNSPCs on the PPP, we incubated cells with 6-aminonicotinamide (6-AN; 500 μ M; Fig. 2d) in the presence of glucose (25 mM). 6-AN blocks entry of glucose-6-phosphate into the PPP by inhibiting 6-phospho-gluconate dehydrogenase, but does not affect anaerobic glycolytic ATP synthesis. Following 24 h incubation with 500 μ M 6-AN, eNSPC survival was considerably impaired (by approximately 60%), whereas the survival of cortical neurons was narrowly affected. Adult NSPC viability was also impaired in the presence of 6-AN (500 μ M; Figure S1). The low dose 6-AN toxicity suggests that eNSPCs require the PPP for survival and/or continued proliferation.

Lactate production, LDH activity, and glucose consumption are up-regulated in eNSPC cultures

A high rate of anaerobic glycolytic flux is associated with increased conversion of pyruvate into lactate. Cells with high glycolytic flux generally display high enzymatic activity of LDH. As an additional assessment of eNSPC glycolytic state under normoxic conditions, we measured lactate concentration within media conditioned by eNSPCs versus neurons and also measured intracellular LDH activity. As shown in Fig. 3a and b, lactate production and LDH activity were increased by 1.5- and 2-fold in eNSPCs compared to those of primary cortical neurons, respectively, when cultured under normoxic conditions. To assess changes in glucose consumption in eNSPCs compared to neurons, we measured glucose levels in initially prepared media and that which was conditioned by both cell types. Glucose consumption from the initial media was elevated by approximately 4.4-fold in eNSPC cultures compared to neuronal cultures ($7.51 \pm 0.83\%$ vs. $1.71 \pm 0.86\%$, eNSPCs vs. neurons, respectively; Fig. 3c). These data are consistent with a higher rate of glycolytic flux in eNSPCs under normoxic conditions.

Structural integrity of mitochondria

Mitochondria within some types of somatic stem cells display immature structural features (e.g., spherical mitochondria with underdeveloped cristae), which has been linked to low mitochondrial oxidative phosphorylation (Chung *et al.* 2007). Many studies have found that low oxygen consumption in various stem cell populations is associated with additional aspects of low mitochondrial function, including decreased mitochondrial number, decreased mitochondrial maturation, and/or reduced synthesis of electron transport proteins (Takubo *et al.* 2010; Takubo and Suda 2012; Zhou *et al.* 2012). It has recently been described that NSPCs isolated from the SVZ of aged mice display decreased mitochondrial content and lower oxidative metabolism than that of young mice (Stoll *et al.* 2011).

To determine whether eNSPCs in our studies harbor functional mitochondria, cells were stained using MitoTracker® Red (Invitrogen) which accumulates in active mitochondria. As shown in Fig. 4a, eNSPCs are fluorescently labeled with MitoTracker Red®. Furthermore, transmission electron microscopy revealed elongated mitochondria within eNSPCs with typical cristae structure (Fig. 4b). NSPC mitochondrial ultrastructure can be compared to that of cortical neurons published in a recent report (Robert *et al.* 2012). These data do not reveal apparent morphological evidence to indicate impaired mitochondrial function.

Deletion of *Hif1a* improves the ability of eNSPCs to utilize pyruvate during glycolytic inhibition, but does not render eNSPCs susceptible to prolonged hypoxia

HIF-1 α is known to promote a glycolytic metabolic state through HIF-1-mediated up-regulation of glycolytic enzymes and down-regulation of enzymes that drive use of mitochondrial metabolism (Semenza 2011). To explore whether the metabolic phenotype we observed for NSPCs is regulated by HIF-1 α , we isolated NSPCs from *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{wt/wt} and *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{-/-} embryonic mice (see Methods section). No significant difference was observed between eNSPCs isolated from *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{wt/wt} versus *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{-/-} when cells were treated for 24 h with: anoxia (< 0.2% O₂; Fig. 5b), cyanide (1 mM; Fig. 5c), 6-AN (500 μ M; Fig. 5d), or galactose (25 mM; Fig. 5e). However, when eNSPCs were treated with 2-DG (10 mM; in the presence of 10 mM glucose), *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{-/-} eNSPCs displayed a 2-fold higher survival in the presence of 0.1 mM pyruvate than *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{wt/wt} eNSPCs (Fig. 5f). These experiments were repeated in NSPCs isolated from adult brain (Figure S3). We found that *Hif1a* gene deletion does not alter adult NSPC viability, as seen for the embryonic cells, and adult NSPCs remain insensitive to pyruvate replacement of glucose. These data suggest that HIF-1 α expression does not underlie the ability of NSPCs to withstand prolonged hypoxia, and only moderately improves pyruvate utilization under conditions of glycolytic inhibition.

To determine whether the loss of HIF-1 α is compensated for by its paralog HIF-2 α , we measured HIF-2 α protein levels in *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{wt/wt} versus *nestin*-CreER^{T2}:R26R-YFP: *Hif1a*^{-/-} cultured eNSPCs. Because HIF-2 α has been shown to play a functional role in malignant glioma (Li *et al.* 2009; Zhou *et al.* 2011), we compared HIF-2 α protein expression to that of the U87 malignant glioma line. We found that HIF-2 α protein

levels in our eNSPCs are dramatically lower than that of glioma cells, and do not increase when *Hif1a* is deleted (Figure S4).

Discussion

This study demonstrates robust survival of NSPCs under conditions of anoxia and mitochondrial inhibition, with increased importance of glycolysis for survival compared to primary cortical neurons. Interestingly, the high requirement for glycolysis in NSPCs appears linked to reliance on the PPP for meeting metabolic needs, and not entirely for the production of aerobic glycolytic ATP. Using an inducible Cre/loxP approach, we also show that sustained deletion of exon 1 of the *Hif1a* gene within undifferentiated NSPCs moderately increases mitochondrial pyruvate utilization, but has no effect on other parameters of metabolism that were tested here, including the ability of NSPCs to withstand prolonged anoxia. Taken together, these data not only confirm that cultured NSPCs have a relatively low requirement for oxidative metabolism for their survival but also demonstrate that this phenotypic trait is not dependent upon HIF-1 α signaling under our culture conditions.

Low oxidative metabolism in NSPCs might be linked to hypoxic niche environments *in vivo*. It has recently been reported that tissue O₂ in adult mouse hippocampal dentate SGZ under non-pathological conditions is less than 1.3%, suggesting a low O₂ NSPC niche environment (Mazumdar *et al.* 2010). Similarly, embryonic NSPCs exist in a low oxygen environment *in utero*, where the fetal cortex has an estimated tissue O₂ of ~1.3% (Bishai *et al.* 2003). Although the adult SVZ is highly vascularized, cellular oxygen levels within the SVZ have been estimated to be only ~2.5–3.0% under non-pathological conditions (Santilli *et al.* 2010), and fall to < 1.3% following focal cerebral ischemia of middle cerebral artery in rat (Thored *et al.* 2007). Cultured NSPCs from both rodent and human have been shown to survive best when maintained under reduced oxygen tensions, with enhanced proliferation and decreased cell death under conditions of physiological oxygen tensions, that is, 3–5% O₂ (Studer *et al.* 2000; Pistollato *et al.* 2007; Panchision 2009). Our results demonstrate that NSPCs display robust survival even under conditions of pathological hypoxia (< 0.1% O₂) and under conditions of mitochondrial inhibition, but not under conditions of impaired glycolysis. Our results are in agreement with a previous report where it was demonstrated that NSPCs from adult brain withstand 24 h of anoxia, but not conditions of glycolytic inhibition in the presence of pyruvate (Burgers *et al.* 2008), and also with a more recent report of decreased oxygen metabolism and mitochondrial function in NSPCs isolated from aging brain (Stoll *et al.* 2011).

Although NSPCs are capable of withstanding anoxia, they display poor survival under conditions of glycolytic inhibition, even in the presence of pyruvate and ample oxygen. The glycolytic requirement of NSPCs does not appear to be entirely because of their need for glycolytic ATP production, but rather because of their dependence on glycolytic intermediates to meet metabolic needs. For example, we found that NSPCs grown in galactose medium display impaired viability compared to neuronal cultures, but only by 20–25%. Since conversion of galactose to pyruvate via glycolysis yields no net ATP, it appears that NSPCs are capable of utilizing mitochondrial oxidative phosphorylation sufficient to

meet most of their ATP demand if required to do so. One difference between glycolytic inhibition with 2-DG (which leads to 60–100% loss of viability) and growth in galactose medium (which leads to 20–25% loss in viability) is that galactose enters glycolysis at glucose-6-phosphate and thereby leaves the PPP intact, but its metabolism does not produce a net increase in glycolytic ATP production, whereas 2-DG inhibition of glycolysis impairs both PPP and glycolytic ATP production. A major function of high glycolytic rate under conditions of normoxia within proliferating cells is to support macromolecular synthesis and other anabolic processes [reviewed in (Lunt and Vander Heiden 2011)]. A high rate of glycolytic flux in NSPCs is also suggested by their relatively high LDH activity, lactate production, and glucose consumption compared with cortical cultures, where the glucose consumption of eNSPCs was similar to what has been described in glycolytic cancer cells with a similar amount of lactate production (Zhou *et al.* 2011). Shunting of pyruvate into lactate generates NAD⁺ required for continued glycolysis (Lunt and Vander Heiden 2011). Thus, it appears that NSPCs may be capable of utilizing mitochondrial oxidative phosphorylation sufficient to meet most of their ATP demand if required to do so, but complete glycolytic inhibition is further detrimental because of the need for glycolytic intermediates to support anabolic processes.

A caveat to the presented experiments is that both neurons and NSPCs were cultured in media containing 25 mM glucose, the concentration of glucose usually used in Dulbecco's modified Eagle's medium and Neurobasal medium (Brewer 1995). However, blood glucose levels *in vivo* range from 8 mM in the human (MacKay 1932) to 10 mM in the rat (Sun *et al.* 2003). High glucose levels in cultured cells may contribute to the Crabtree effect, where cells shift ATP production away from the electron transport chain toward glycolysis because of the presence of excess substrate (Frezza and Gottlieb 2009). However, neurons require 25 mM glucose to survive in culture (Russell *et al.* 2002), and appeared to maintain oxidative metabolism in our studies. Future studies to determine whether NSPCs succumb to the Crabtree effect could be resolved by testing the metabolic phenotype of NSPCs under lower concentrations of glucose. Regardless, our studies show that when neurons and NSPCs are cultured using the same concentration of glucose their metabolic phenotypes differ.

It was somewhat surprising that HIF-1 α does not appear to be the primary metabolic regulator of glycolytic and oxidative metabolism within NSPCs under standard culture conditions. Many studies have linked HIF-1 α regulation of metabolic state to pluripotency in various stem cell populations, including human embryonic stem cells (hESCs) and bone marrow hematopoietic stem cells (HSCs). For example, hESCs are almost exclusively glycolytic, even when cultured in the presence of ample oxygen (Zhou *et al.* 2012). The restricted oxidative capacity in hESCs has been attributed to low numbers of mitochondria and to suppressed activity of mitochondrial complex IV cytochrome c oxidase linked to constitutively high HIF-1 α expression (Zhou *et al.* 2012). Similarly, primitive HSCs freshly isolated from bone marrow utilize glycolysis instead of mitochondrial oxidative phosphorylation to meet their energy demand, and can be isolated by flow cytometry based solely on their low mitochondrial potential (Simsek *et al.* 2010). These primitive HSCs are primed for hypoxic resistance and express high levels of HIF-1 α (Simsek *et al.* 2010). Selective and inducible deletion of exon-1 of the *Hif1a* gene in the HSC population *in vivo*

results in loss of the quiescent HSC population and loss in the ability of these cells to repopulate recipient bone marrow following serial transplantation (Takubo *et al.* 2010).

Although our results suggest that HIF-1 α expression is not linked to metabolic phenotype of cultured NSPCs, this does not necessarily imply that *Hif1a* gene expression is not important for metabolic phenotype and maintenance of the stem cell population *in vivo*. We previously demonstrated constitutive *Hif1a* gene expression within the nestin-positive NSPCs of adult SVZ and SGZ *in vivo* (Roitbak *et al.* 2011). Further, inducible *Hif1a* gene deletion in adult *nestin-CreER^{T2}/R26R-YFP/Hif1a^{f1/f1}* mice depletes primitive neural stem cells and downstream transient amplifying progenitors within the SVZ, although this may be secondary to regression of SVZ vasculature and/or impairment of other intracellular signaling mechanisms (Cunningham, unpublished observations). Similarly, it has recently been reported that *Hif1a* gene deletion in adult CNS impairs hippocampal neurogenesis, mediated through impairment of Wnt/ β -catenin signaling rather than via regulation of metabolic phenotype (Mazumdar *et al.* 2010).

Maintenance of NSPCs under high growth factor stimulation in culture, as done here, might override normal HIF-1 α signaling. For example, growth factor signaling, tyrosine kinase activity, and PI3K/Akt activity have been shown to up-regulate the glycolytic phenotype through modulation of glycolytic enzymes, as well as stimulation of glucose uptake and utilization (Vander Heiden *et al.* 2009). EGF stimulates cultured NSPCs to proliferate, and selects for rapidly proliferating transient amplifying progenitor cells and not the quiescent primitive stem cell population that maintains the NSPC pool *in vivo* (Pastrana *et al.* 2011). Growth factors also promote glycolysis and lactate production by signaling through PI3K/Akt to up-regulate protein synthesis via mTOR of glucose transporters and glycolytic enzymes independent of HIF-1 α signaling (DeBerardinis *et al.* 2008).

In summary, our findings indicate a high innate resistance of NSPCs to anoxic conditions in culture that does not require HIF-1 α signaling. *Hif1a* gene deletion within NSPCs also did not stimulate a major shift from glycolytic dependence and therefore is unlikely to regulate this metabolic phenotype. Future studies will be required to elucidate the role of HIF-1 α on metabolic signaling within NSPCs *in situ*, particularly with respect to their ability to withstand metabolic injury and mount regenerative responses that promote functional recovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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acquired by KMC. The article was drafted by KMC and LAC, and critically revised by KMC, CWS, and LAC. The final version of the manuscript was approved by KMC, CWS, and LAC.

Abbreviation used

HIF-1α	hypoxia inducible factor-1alpha
HSCs	hematopoietic stem cells
NSPCs	neural stem/progenitor cells
PPP	pentose phosphate pathway
SGZ	subgranular zone
SVZ	subventricular zone

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Fig. 1.

Embryonic neural stem/progenitor cells (NSPCs) are more resistant than neurons to 24 h anoxia. (a) Viability of oxygen-deprived (< 0.2% O₂; 24 h) neurons and eNSPCs was measured using calcein AM fluorescence and compared as a percent of normoxic (21% O₂) controls. (b–e) Micrographs imaged at 10 × of neurons (b, c) and eNSPCs (d, e) exposed to normoxic (b, d) or anoxic (c, e) conditions for 24 h. Neurons are labeled with NeuN (b, c); eNSPCs are labeled with nestin (d, e). Scale bar = 500 μm. *n* = 3 per treatment group. ****p* < 0.001.

Fig. 2.

Embryonic neural stem/progenitor cells (NSPCs) are more resistant to cytochrome c oxidase inhibition but more susceptible to glycolytic and pentose phosphate pathway inhibition than neurons. (a) Viability of cyanide-exposed (1 mM; 24 h) neurons and eNSPCs as measured using calcein AM fluorescence and compared as a percent of untreated controls. (b) Neurons and eNSPCs were treated with 10 mM 2-deoxy-D-glucose (2-DG) and 10 mM glucose, in the presence of increasing concentrations of pyruvate (0.1–10 mM) for 24 h. Viability of neurons (open circles) and eNSPCs (closed circles) as compared as a percent of controls that were not treated with 2-DG. Two-way ANOVA shows a significant difference between cell type ($p < 0.0001$) and concentration of pyruvate ($p < 0.001$). (c) Viability of neurons and eNSPCs treated with 25 mM galactose as measured using calcein AM fluorescence and compared as a percent of glucose-treated (25 mM) controls. (d) Viability of neurons and eNSPCs exposed to 500 μ M 6-AN for 24 h as compared to vehicle (dimethylsulfoxide) controls. $n = 3$ per treatment group. * $p < 0.05$. *** $p < 0.001$. **** $p < 0.0001$.

Fig. 3.

Embryonic neural stem/progenitor cell (NSPC) cultures display elevated lactate production, lactate dehydrogenase (LDH) activity, and glucose consumption compared with neuronal cultures. Cells were cultured in phenol-free growth medium for 24 h. (a) Lactate concentration as measured in incubated media of neurons versus eNSPCs. (b) Intracellular lactate dehydrogenase (LDH) activity as measured in neurons versus eNSPCs. (c) Glucose consumption by cultured neurons versus eNSPCs. The amount of glucose was measured in initial culture media (control) and compared to the amount of glucose in conditioned media following 24 h incubation with neurons versus eNSPCs. $n = 4$ per treatment group. * $p < 0.05$. ** $p < 0.01$. **** $p < 0.0001$.

Fig. 4. Embryonic neural stem/progenitor cells (NSPCs) display mature mitochondria. (a) Micrograph of eNSPCs stained with MitoTracker[®] Red reveals the presence of mitochondria (60 ×). Scale bar = 20 μm. (b) Mitochondrial morphology as viewed by transmission electron microscopy. Scale bar = 250 nm.

Fig. 5.

Deletion of hypoxia inducible factor-1alpha (*Hif1a*) does not alter the metabolic phenotype of embryonic neural stem/progenitor cells (NSPCs). (a) PCR of genomic DNA showing successful recombination between the loxP sites in the *Hif1a* gene from YFP+ *nestin*-CreERT2: R26R-YFP:*Hif1a*^{Δ/Δ} eNSPCs (*Hif1a*^{Δ/Δ}). (b–f) 24 h treatment of *nestin*-CreERT2:R26R-YFP:*Hif1a*^{wt/wt} (*Hif1a*^{wt/wt}) versus *nestin*-CreERT2: R26R-YFP:*Hif1a*^{Δ/Δ} (*Hif1a*^{Δ/Δ}) eNSPCs isolated from mice exposed to (b) anoxia, (c) CN (1 mM), (d) 6-AN (500 μM), (e) galactose (25 mM), or (f) 2-DG (10 mM) plus glucose (10 mM) and pyruvate (0.1–10.0 mM). Two-way ANOVA shows a significant difference between genotype (**p* < 0.05) and concentration of pyruvate (***p* < 0.01) for (f). *n* = 3 per treatment group.